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14. ABSTRACT

As a result of androgen ablation TGF-ß1 expression levels transiently elevate and regression of benign prostate hyperplasia as well as prostate cancer cells for the most part occur. Better understanding of prostate androgen responsiveness is critical in understanding and ultimately combating androgen-non-responsive prostate cancer. Studying the conditional TGF-ß type II receptor fibroblast knockout mouse model we developed (FßKO), we found that TGF-ß signaling in the prostate stromal fibroblasts regulate both stromal and epithelial differentiation in the prostate. As proposed we attempted to develop mice that are stromally knocked out for TGF-ß signaling and express the large Tantigen in the prostate epithelia, but was unsuccessful. Thus we made tissue recombinants of prostatic epithelia with FßKO stromal cells. This resulted in the development of poorly differentiated adenocarcinoma compared to when the same epithelia was combined with control stromal cells. Moreover, we found that the FßKO associated epithelia was refractile to androgen ablation. The mechanism of these observations seems to be due to stromally derived paracrine Wnt5a activating the epithelial frizzled receptor 2 that enabled prostate epithelial survival in an androgen deficient environment. We hope to gain permission to progress with these experiments to further address the mechanism of stromal TGF-ß signaling impact on prostate cancer androgen responsiveness and differentiation.

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INTRODUCTION

The prostate epithelial and stromal compartments interact to regulate prostate development and function, in part through the tight regulation of glandular apoptosis and proliferation. These interactions are mediated by various factors that include hormones and cytokines. Specific signaling by androgens is required for prostate development and maintenance of function through the stimulation of proliferation and inhibition of apoptosis of prostatic epithelial cells (Hayward and Cunha, 2000; Montgomery et al., 2001). Cytokines such as EGF, IGF, and TGF-ß isoforms can also in-turn stimulate the expression of the androgen receptor (AR) in an androgen-independent fashion (Byrne et al., 1996; Culig et al., 1996). Often the development and progression of prostate cancer is dependent on androgens and their receptor for prostate cellular proliferation and differentiation. As a result, its inhibition has been the primary therapy for metastatic prostate cancer and much effort has been devoted to elucidating the role of the androgen receptor in prostate cancer. As a result of androgen ablation TGF-ß1 expression levels transiently elevate and regression of benign prostate hyperplasia as well as prostate cancer cells for the most part occur. Better understanding of prostate androgen responsiveness is critical since androgen-signaling antagonists are currently used in treating patients with malignant prostate cancer, benign prostate hyperplasia, and as a chemoprevention of prostate cancer in clinical trials. However populations of hormone-non-responsive cancer cells unfortunately frequently arise. The **central hypothesis** of this proposal is that TGF-ß signaling in the prostatic fibroblasts contributes to normal prostatic epithelial differentiation; when this signal is altered in the case of some cancers the differentiation status of the epithelia is altered.

TGF-ß isoforms (TGF-ß1, ß2, ß3) have long been established as physiological regulators of prostate growth because of their ability to inhibit cell proliferation and mediate apoptosis (Kyprianou and Isaacs, 1989; Martikainen et al., 1990). TGF-ßs exert their effects through binding to the TGF-ß type II receptor (TßRII) and subsequent recruitment of the type I receptor (TßRI) for downstream cytoplasmic signaling through multiple parallel signaling pathways (Attisano and Wrana, 2002). TGFß plays a key role in the steroidal regulation of tissues and in the important growth regulation axis existing between androgenic signaling, smooth muscle differentiation and epithelial proliferation. The present proposal seeks to address the role of TGFß signaling in mouse prostate tissue under in vivo conditions in light of signaling pathways identified through studies in cell lines. In order to understand the role of TGF-ß in the prostate we have developed and studied mouse models, that employ the Cre-lox methodology to conditionally ablate TßRII expression in the stromal fibroblasts (Bhowmick et al., 2004) and those that express the large SV40 T antigen transgene (TAG) in the prostate epithelia (from collaborator, Dr. Robert Matusik, Vanderbilt U., TN) (Kasper et al., 1998). The proposal was based on preliminary data that ablation of TßRII in fibroblasts results in preneoplastic prostate intraepithelial neoplasia (PIN) lesions and prostate-specific TAG expression results in PIN and progression of focal adenocarcinoma (Kasper et al., 1998). We proposed to develop mice that expressed both TAG in the prostate epithelia and concomitant loss of TßRII in the stromal fibroblasts (mouse model termed TNT) to examine epithelial and stromal differentiation (Task1). Since these TNT mice were not thought not to be able to live past 8 weeks of age, we also proposed to rescue the prostatic tissues from these mice as xenografts and further study differences in cellular differentiation and androgen responsiveness (Task2).

BODY

We focused on how the TGFß signaling pathway components may be involved in prostate cancer progression and subsequent regression. It was not feasible to study prostate cancer progression in the mouse model that expressed both TAG in the prostate epithelia and concomitant loss of TßRII in the stromal fibroblasts (mouse model termed TNT) due to early lethality (Task1). Previously, we developed the capacity to culture prostatic fibroblasts from wild type and FßKO (fibroblastic knockout for the TGFß receptor) mice. This allowed us to perform tissue recombinant xenografts where wild type epithelial cells were recombined with the two stromal cell types. We found that the combination of FßKO stromal cells with wild type epithelial cells resulted in the development of PIN lesions in (transformation) in the epithelial compartment with in 8 weeks of xenografting (**Figure 1**). The similar recombination with wild type stromal cells resulted in normal prostatic structures in the subrenal xenografts. These results suggest that the loss of stromal TGFß responsivity results in the transformation of prostatic epithelia independent of other factors associated with the FßKO intact mice. Interestingly, prostates from FßKO mice do not seem to progress from PIN to prostate adenocarcinoma. Thus it was important to understand the role of TGFß signaling by the stromal cells in the context of epithelia mutations that can result in adenocarcinoma.

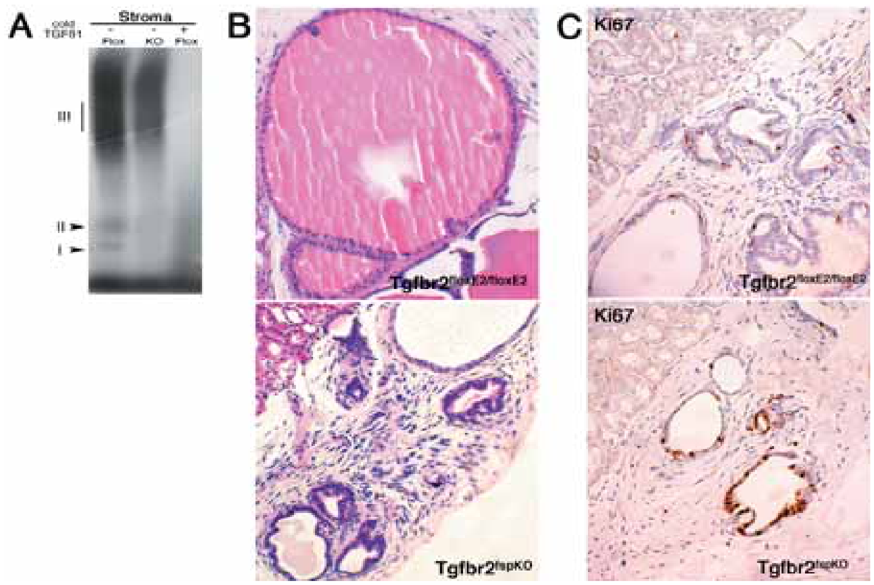
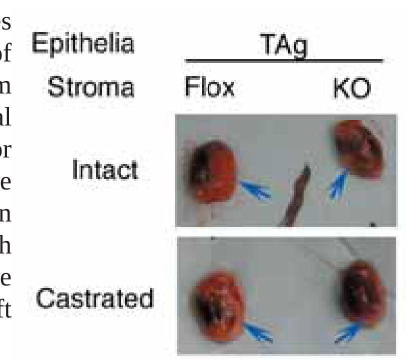


Figure 1. The conditional knockout of Tgfbr2 through the targeting of Cre expression by the FSP1 promoter results in recombination of Tgfbr2$^{flox/flox}$ in stromal fibroblasts through out the mouse. (A) Primary cultures of prostate epithelia and fibroblasts from Tgfbr2$^{floxE2/floxE2}$ (Flox) and Tgfbr2^{fspKO} (KO) mice were affinity cross-linked with [^{125}I]-TGF-ß1 in the presence or absence of competing cold TGF-ß1. (B) The recombination of wild type epithelial organoids with either Tgfbr2$^{floxE2/floxE2}$ or Tgfbr2^{fspKO} result in the prostatic glandular formation in sub-renal xenografts. The Tgfbr2^{fspKO} stroma induced normal prostatic epithelia to form PIN like structures compared to those associated with Tgfbr2$^{floxE2/floxE2}$ stromal cells. (C) Ki67 immunostaining suggest elevated proliferation of the prostatic epithelia associated with Tgfbr2^{fspKO} stromal cells.

The expression of the large T antigen by the prostatic epithelia, driven by the probasin promoter in transgenic mice, results in primarily in PIN lesions by 12-15 week of age with occasional foci of adenocarcinoma. Initial studies in recombining prostatic stromal cells from Flox and FßKO mice with the 12T7f epithelial organoids proved to be interesting. The recombined tissues were xenografted under the renal capsule for 8 weeks. After which time, some of the host mice were castrated. **Figure 2** shows the gross images of the tumor tissues from these experiments. Interestingly we found that the FßKO stroma resulted in smaller tumors, however the epithelia associated with the FßKO stromal cells were less differentiated than those associated with wild type stromal cells (**Figure 3**). The gross image in **Figure 2** also suggested FßKO stromal cells inhibited epithelial of regression upon castration. The staining for TUNEL provided indices for apoptosis following castration of the host mice (**Figure 4**). This phenotype is reminiscent of that observed in the FßKO mice. Thus together it would suggest that the stromal cells mediate the size and androgen responsivity of the prostate ans well as prostate cancer.

Next we wanted to determine the mechanism for the phenotype observed with regard to the lack of regression following castration. In doing so we found that Wnt signaling was upregulated by the FßKO stroma compared to the Flox stroma *in vivo*. Through real time

Figure 2. Gross pictures of tissue recombinations of prostatic epithelial cells from LADY TAg mice with stromal cells from Flox (control) or FßKO mice 5 weeks. The lower panel shows regression of the tumor associated with the Flox stromal cells. The arrows indicate the xenograft within the renal capsule.



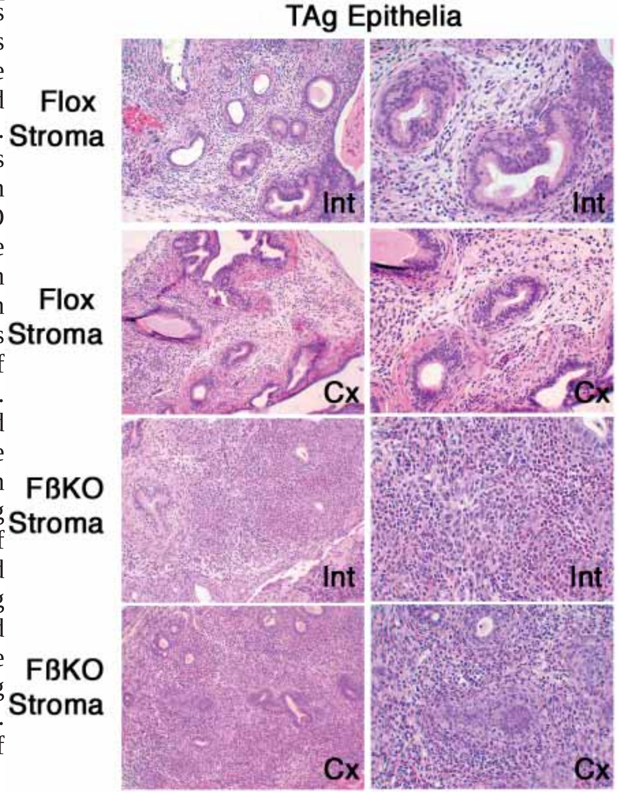


Figure 3. Tissue recombinants of Flox or FßKO stromal cells with epithelia expressing the large T antigen were xenografted for 9 weeks in SCID mice. The histology indicated less differnetiated epithelia when associated with the FßKO stroma compared to the PIN like lesions when associated with Flox stromal cells. Androgen responsivity of the grafts was determined by castration of the host mice for seven days. The resulting ducts associated with Flox stromal cells were appreciabbbly smaller than those from the corresponding intact mice. The duct size of the FßKO associated grafts did not seem to change following castration. The left (200x) and right (400x) panels are indicate xenografts from hosts following castration (Cx) or intact (Int). See Figure 2 for gross images of the tissues shown here.

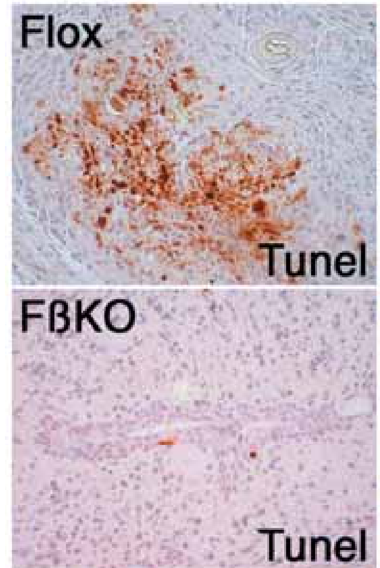


Figure 4. Apoptosis of the tumors were greater following castration when Flox stromal cells were used in contrast to when FßKO stromal cells were used. TUNEL staining (brown) of the xenografts shown in Figures 2 and 3 indicate apoptotic index of the tumors seven days following castration.

RT-PCR of prostates we found that there were elevated levels of Wnt5a expression intact FßKO prostates compared to age matched at 6 week control prostates of Flox mice (**Figure 5**). The Wnt5a-Frizzeled receptor complex stimulates ß-catenin stabilization and nuclear translocation coupled to TCF-4 (T cell factor). This transcription factor complex binds promoters of various genes that include those of c-myc and cyclinD1. The TCF/ß-catenin consensus binding sequence has been incorporated into an artificial reporter construct termed TOP by the Volegstein laboratory for assays in cultured cells (He et al., 1998). However, to better examine canonical Wnt signaling *in vivo*, a transgenic mouse termed TOPGal was developed by the Fuches laboratory with a similar TOP reporter driving the bacterial LacZ gene to allow visualization of promoter activity through ß-galactosidase blue staining (DasGupta and Fuchs, 1999). Thus, to visualize TGF-ß regulated paracrine Wnt activity in the prostate we crossed the FßKO mice with TOPGal mice. We further, examined canonical Wnt signal activation in the presence and absence of androgen signaling through castration experiments. We found that the Flox-TOPGal (control) mice did not exhibit ß-galactosidase activity in the intact mice, but was active in a fraction of the prostatic luminal epithelials cells from days 3-5 following castration (**Figure 6**). Interestingly, there was no visible ß-galactosidase activity 7 days following castration in Flox/TOPGal prostates (data not shown). In contrast, FßKO/TOPGal prostatic epithelia exhibited Wnt activity in intact mice and through the seven days following castration (**Figure 6**). These results were confirmed by examining Wnt5a expression in the stromal compartment of laser captured paraffin embedded prostatic tissues from intact and castrated Flox and FßKO mice (data not shown). The fact that only a faction of the regressing Flox/TOPGal prostatic epithelia exhibited TOP activity, yet the entire non-regressing FßKO/TOPGal prostate have TOP activity suggest that Wnt signaling may be a survival signal.

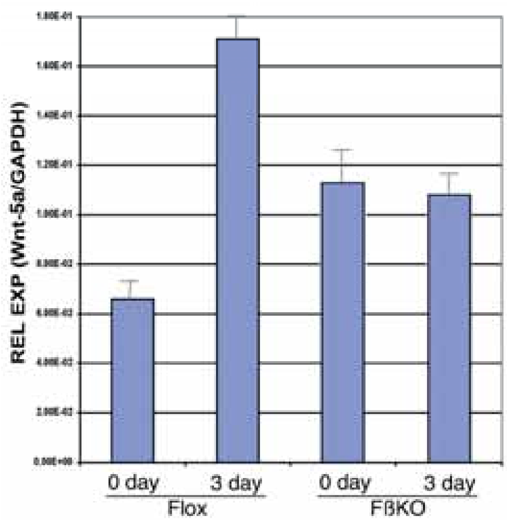


Figure 5. The expression of Wnt5a was determined by real time RT-PCR of prostates from Flox and FßKO mice. RNA was extracted from whole dorsolateral lobes from intact (0 day) or 3 days following castration. The amplified cDNA was measured by Syber Green fluorescence on a BioRad ICycler PCR machine..

These results lead us to suggest the hypothesis that localized TGF-ß activation following androgen ablation is not in fact a direct signal for apoptosis, but rather a inhibitor of a survival signal mediated through epithelial

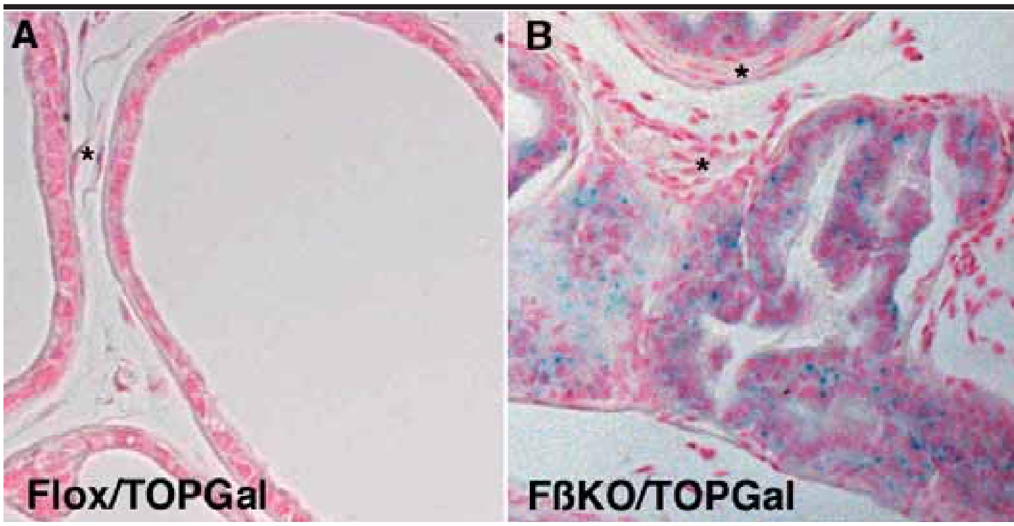


Figure 6. Transgenic Flox/TOPGal (a) and FßKO/TOPGal (b) mice were developed by crossing the Tgfbr2^{fspko} and TOPGal mice. The prostates of these mice were incubated with X-gal substrate for visualization of Wnt signaling activation, interestingly only in the epithelial compartment of the FßKO/TOPGal prostates. Nuclear fast red was used as a counterstain. Asterisks indicate stromal cells in both panels.

canonical Wnt5a signaling . Thus we develop another model to rescue the lack of androgen responsivity of the FßKO prostates. We chose to inhibit Wnt5a signaling by the frizzled receptor 2 through the expression of the secreted soluble domain of the frizzled receptor 2 (SFzR2) through adenoviral infection. We were unsuccessful in accomplishing this in intact mice, since the FßKO mice were too fragile for the multiple survival surgeries. Thus we harvested prostate tissue from Flox and FßKO mice, incubated with either GFP (control) or SFzR2 adenovirus, and rescued the tissue by xenografting under the renal capsule of syngenic male Bl/6 mice. After 2 weeks the host mice were either castrated or left intact. **Figure 7** shows that FßKO prostates that were infected with SFzR2 adenovirus had increased apoptosis seven days following castration. In comparison GFP transduction did not seem to alter the apoptosis rate on either the Flox or FßKO prostate tissue from what observed in previous experiments where the intact mice were used (see original proposal). We will have a pathologist score the previously stained tissue sections for apoptosis and proliferation. The data will undergo biostatistical evaluation. These striking results of paracrine Wnt5a signaling regulating prostate regression is currently being written up for manuscript submission.

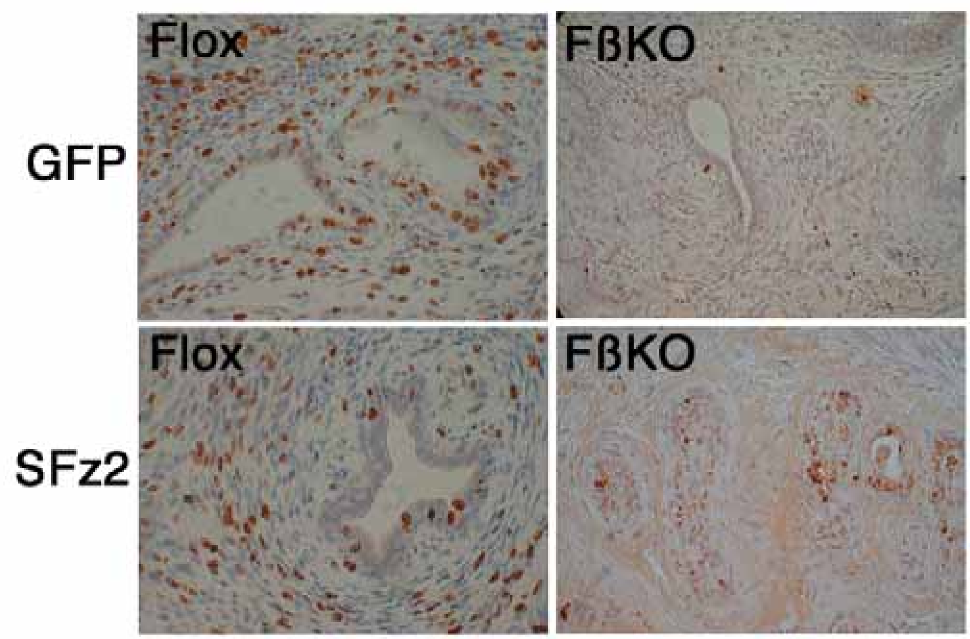


Figure 7. The intact prostates from the Flox and FßKO mice were rescued and xenografted under the renal capsule of syngeneic Bl/6 mice. At the time of xenografting, the tissues were either transduced with adenovirus for the expression of GFP (control) or oluble frizzled receptor 2 (SFzR2). Two weeks after xenografting the the host mice were androgen ablated through the castration and the grafts collected after seven days. TUNEL staining (brown nuclei) illustrates elevated apoptosis in FßKO following expression of SFzR2. This would suggest canonical Wnt signaling is important in the maintenance of prostatic epithelia following castration.

c. Significance: Surgery and androgen ablation therapy remains the major treatment for prostate cancer. However, within a year of treatment >80% of prostate cancer becomes androgen independent as a result of documented mutations in AR (50%) and unknown factors (50%). New treatments are required that are more effective irrespective of the structure of the AR in the cancerous epithelial cells. Based on our analysis of the Tgfbr2^{fspko} mouse model, we believe that targeting therapy to the prostate stroma rather than the cancer cells may be prudent in patients' androgen non-responsive prostate cancer. The non-transformed stromal cells are likely to have greater genomic stability and are less likely to be subject of mutation adaptation since the target would be the secretion of paracrine factors that would act on the epithelia.

d. Plans: Presently, we have been able to maintain that schedule and we are making every attempt to complete all the tasks proposed.

KEY RESEARCH ACCOMPLISHMENTS

- We provide in vivo data supporting stromal TGF-ß signaling can regulate prostate cancer androgen responsiveness in a tissue recombination model.
- We showed that Wnt5a is expressed by the prostatic stroma following androgen ablation in a short window.
- The activation of Wnt5a is regulated by TGFß responsivity of the prostatic stroma
- Specific inhibition of the Frizzed receptor 2 in the epithelial compartment can result in regression of prostates that were otherwise refractile to androgen ablation.

REPORTABLE OUTCOMES

Research

Manuscripts

None

Abstracts

- Placencio, V.R., Sharif-Afshar, A.-R., Simon W. Hayward, and **Bhowmick N.A.** (2005) Prostatic Epithelial Transformation and Androgen Responsiveness Regulated by Stromal-Epithelial Interactions. (oral presentation) Society For Basic Urologic Research Meeting. Miami, FL.

Awards received based on work supported by this grant

None

Products

CDNA construct, cell lines, and animal models developed

- Development of a *in vivo* tissue recombination model that incorporates large T antigen expressing prostatic epithelia with stromal cells deficient in TGFß responsivity.
- Transgenic mouse line termed FßKO/TOPGal that enables the study of canonical Wnt signaling in the context of stromal cells deficient for TGFß responsivity.

CONCLUSION

The differentiation of the prostatic epithelium and stroma occur concurrently in an androgen-dependent mechanism. The concurrent loss of stromal TGFß responsivity and development of PIN (through targeted expression of the large T antigen) can result in the undifferentiated adenocarcinoma. Further, the TGF-ß signaling in the prostate stroma can dictate androgen responsiveness of the carcinoma cells. To examine the mechanism of these observations, we found that stromally derived paracrine Wnt5a mediates prostate epithelial survival in an androgen deficient environment.

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